5 DETECTION OF BLOOD

5.1 GOALS

5.1.1 To develop a basic understanding of the use of presumptive and confirmatory tests.

5.1.2 To develop a thorough understanding of the procedures used by the Department.

5.1.2.1 To become acquainted with the sensitivity and stability of reagents.

5.1.2.2 To determine the specificity and limitations of the various methods.

5.1.2.3 To acquire a thorough understanding of the use of controls.

5.2 TASKS

5.2.1 Prepare reagents used for the Phenolphthalein, Tetramethybenzidine, and Luminol tests.

5.2.2 Perform the Combined Phenolphthalein -Tetramethylbenzidine (PTMB) and Luminol chemical color tests on the following (record the results for the individual Phenolphthalein and Tetramethylbenzidine reactions):

5.2.2.1 Bloodstains of varying dilutions prepared in normal saline (1:10, 1:100, 1:250, 1:500, 1:750, 1:1000, 1:2000).

5.2.2.1.1 Normal Saline (0.9%):

- 9 g Sodium chloride
- 1000 ml Distilled water
- Mix thoroughly until dissolved.

5.2.2.2 Minimum of 5 bloodstains of varying ages.

5.2.2.3 Minimum of 20 bloodstains subjected to various contaminants (including, but not limited to, super glue, fingerprint powder, ninhydrin, redwop powder - rhodamine base, bleach, soap, motor oil, luminol, and mold), and environmental conditions (heat, moisture, heat and moisture combined, decomposition).

5.2.2.4 All substances reported in the literature to give false positive reactions.

5.2.3 Observe specificity and sensitivity of all tests performed. Compare this information to that found in the literature.

5.2.4 Observe and obtain instruction from qualified examiners performing routine examinations of case material.

5.2.5 Test at least 10 unknown stains provided by training coordinator or designee.

5.2.6 Read applicable literature. Refer to Appendix A and Appendix B.

5.3 TRAINING EVALUATION

5.3.1 Knowledge

5.3.1.1 Review of notes in training notebook by training coordinator.

5.3.1.2 Mini-mock trials/oral and practical examinations.

5.3.1.3 Completion of checklist by training coordinator.

5.3.2 Skills

5.3.2.1 Observation by training coordinator or designee.

5.3.2.2 Review of notes in training notebook by training coordinator.

5.3.2.3 Mock trials/oral and practical examinations.

5.3.2.4 Completion of checklist by training coordinator.

5.4 DETECTION OF BLOOD USING CATALYTIC TESTS – TECHNICAL NOTES

5.4.1 Most of the preliminary chemical tests for blood are based on the detection of hemoglobin by detecting its peroxidase-like activity. Ionic iron forms chelate (ring) structures with many organic compounds and very often such iron-chelates possess catalytic activity in oxidation reactions. An example of a biological catalyst is peroxidase which decomposes hydrogen peroxide or organic peroxides to form free hydroxyl radicals. The heme group of hemoglobin possesses peroxidase-like activity which may catalyze this breakdown of hydrogen peroxide. If no other organic oxidizable compound is present, these radicals decompose to form water and oxygen. If a benzidine derivative or phenolphthalin is present, it will oxidize the colorless reagent to form a colored product.

5.4.2 The peroxidase-like activity of hemoglobin operates in both acidic and basic media, while some of the bacterial and plant enzymes (catalases and peroxidases) are more pH dependent. Therefore, the phenolphthalein test, which takes place in basic medium, and the tests using benzidine derivatives, which take place in acid medium, are not redundant. Fast positive reactions obtained with both tests on a red-brown or other appropriately colored substance can be considered very strong evidence (essentially proof for practical purposes) that the substance being tested is blood. The Combined Phenolphthalein-Tetramethylbenzidine (PTMB) Test has been routinely used by the Department for many years to indicate the presence of blood.

5.4.3 Luminol

5.4.3.1 Luminol can be oxidized by heme to a product which luminesces under darkened conditions. This test is very useful in locating “latent” bloodstains, but should only be performed <u>after</u> a visual search has failed to reveal suspected blood. The reagent is applied as a mist from a spray bottle over the item being analyzed.

5.4.3.2 The degree of luminescence is dependent on the substrate and will fade with time, but can be restored with an additional application of reagent mist. This may be particularly useful

for weak stains that require prolonged exposure times to photograph, but care must be taken to avoid diluting the stains with unnecessary repeat spraying. If "latent" blood is suspected on a vertical surface, be prepared to photograph immediately as the spraying may cause the blood to "run" down the surface.

5.4.3.3 When appropriate, the necessary photographic equipment should be available to document any luminescence produced. If photographing, use a ruler with luminescent tape as a scale and 400 ASA film or higher.

5.4.3.4 Once possible blood is located with luminol, the Combined Phenolphthalein-Tetramethylbenzidine (PTMB) Test must be performed. Since other substances are known to react with luminol, blood is not indicated unless the PTMB Test is positive. Luminol will not interfere with this subsequent test.

5.5 PROCEDURES FOR THE DETECTION OF BLOOD

5.5.1 COMBINED PHENOLPHTHALEIN-TETRAMETHYLBENZIDINE (PTMB) TEST (References 2, 3, 4, Appendix B)

5.5.1.1 Safety Considerations

5.5.1.1.1 Phenolphthalin - Caution! Avoid contact and inhalation!

5.5.1.1.2 Potassium hydroxide - Caution! Corrosive! Poisonous!

5.5.1.1.3 Tetramethylbenzidine - Caution! Harmful if swallowed, inhaled or absorbed through skin! Emits toxic fumes under fire conditions!

5.5.1.1.4 Glacial acetic acid - Caution! Corrosive! Flammable!

5.5.1.1.5 Ethanol - Caution! Flammable! Poisonous!

5.5.1.1.6 Oxidized zinc - Caution! Danger of spontaneous combustion if allowed to dry!

5.5.1.2 Materials and Equipment

5.5.1.2.1 Dropper bottles

5.5.1.2.2 Cotton swabs

5.5.1.2.3 Test tubes, microtiter plates, or filter paper

5.5.1.2.4 100 ml graduated cylinder

5.5.1.2.5 Weigh boats or weigh paper

5.5.1.2.6 Balance

5.5.1.2.7 Spatula

5.5.1.2.8 Scissors

5.5.1.2.9 Tweezers

5.5.1.3 Stock Solutions

5.5.1.3.1 Phenolphthalin Stock Solution

- 1 g Phenolphthalin
- 25 g Potassium Hydroxide (KOH)
- 100 ml Distilled water
- The above ingredients are mixed until thoroughly dissolved.

5.5.1.3.1.1 Storage

5.5.1.3.1.1.1 This colorless solution is stored under refrigeration over fresh granular zinc to keep it in the reduced form. The oxidized zinc in the bottle should not be allowed to dry (see 5.5.3.1.6 Safety Considerations and 5.5.3.3.1.3.1 Disposal).

5.5.1.3.1.2 Labeling

5.5.1.3.1.2.1 Label the bottle as Phenolphthalin Stock Solution with a lot number (the date of preparation followed by the initials of the person preparing the stock solution). Example: Phenolphthalin Stock Solution Lot Number 100899JD was prepared by Jane Doe on October 8, 1999.

5.5.1.3.1.2.2 There is no expiration date (see 5.5.3.5 Minimum Standards and Controls).

5.5.1.3.1.3 Disposal

5.5.1.3.1.3.1 When the reduced phenolphthalin stock solution is depleted, cover the zinc in the bottom of the bottle completely with a solution of potassium hydroxide in distilled water (25 g KOH/100 ml dH_20).

5.5.1.3.1.3.2 Label the bottle with the contents and "For Disposal" and refrigerate. Notify the Safety Officer (Eastern, Northern, and Western Laboratories) or the Department Safety Coordinator (Central Laboratory) that the zinc

is ready to be disposed of in accordance with Department procedures.

5.5.1.3.2 Tetramethylbenzidine (TMB) Stock Solution

- 10 mg Tetramethylbenzidine (TMB)
- 30 ml Glacial acetic acid
- Mix the above ingredients until thoroughly dissolved.

5.5.1.3.2.1 Storage

5.5.1.3.2.1.1 The TMB stock solution may be stored under refrigeration or at room temperature.

5.5.1.3.2.2 Labeling

5.5.1.3.2.2.1 Label the bottle as TMB Stock Solution with a lot number (the date of preparation followed by the initials of the person preparing the stock solution).
Example: TMB Stock Solution Lot Number 100899JD was prepared by Jane Doe on October 8, 1999.

5.5.1.3.2.2.2 There is no expiration date (see 5.5.3.5 Minimum Standards and Controls).

5.5.1.3.2.3 Disposal

5.5.1.3.2.3.1 Dispose of the TMB stock solution and other materials contaminated with this solution as hazardous waste in accordance with Department procedures.

5.5.1.4 Working Solutions

- Distilled water
- Ethanol
- 3% Hydrogen peroxide
- 1:5 dilution of phenolphthalin stock solution in distilled water (1 part of the phenolphthalin stock solution diluted with 4 parts of distilled water)
- TMB stock solution

5.5.1.4.1 Storage

5.5.1.4.1.1 All bottles of working solutions are stable at room temperature.

5.5.1.4.2 Labeling

5.5.1.4.2.1 Bottles containing working solutions of ethanol and 3% hydrogen peroxide will be labeled with the contents and the lot number.

5.5.1.4.2.2 The bottle containing the 1:5 dilution of phenolphthalin stock solution must be labeled appropriately with the lot number of the stock solution, the date of the dilution, and the initials of the person making the dilution.

5.5.1.4.2.3 There is no expiration date for the working solutions (see 5.5.3.5 Minimum Standards and Controls).

5.5.1.5 Minimum Standards and Controls

5.5.1.5.1 On the day of use a positive reagent control (known bloodstain) and a negative reagent control (distilled water) must be tested to ensure that the reagents are working properly. The results of this testing must be documented in the case file.

5.5.1.5.2 If either control does not give the expected result, do not proceed with testing evidence samples until the problem has been resolved as demonstrated by testing another set of positive and negative reagent controls and achieving the expected results with both controls.

5.5.1.6 Combined Phenolphthalein-Tetramethylbenzidine (PTMB) Test Procedure

5.5.1.6.1 Gently rub a suspected stain with a cotton swab which has been moistened with distilled water or place a small cutting of the stain in a small test tube or microtiter plate, or on filter paper and moisten with distilled water if desired.

5.5.1.6.2 Add one drop of ethanol.

5.5.1.6.3 Add one drop of 1:5 dilution of phenolphthalin (i.e., the working solution of phenolphthalin).

5.5.1.6.4 Add one drop of 3% hydrogen peroxide.

5.5.1.6.5 Note any color change. An immediate pink color is expected if blood is present.

5.5.1.6.6 Add one drop of tetramethylbenzidine stock solution.

5.5.1.6.7 Note any color change. An immediate blue-green color is expected if blood is present.

5.5.1.6.8 Interpretation

5.5.1.6.8.1	Positive Reaction =	Immediate pink color at 5.5.3.6.5, followed by immediate blue-green color at 5.5.3.6.7
5.5.1.6.8.2	Negative Reaction =	No color change at 5.5.3.6.5, followed by no color change at 5.5.3.6.7
5.5.1.6.8.3	Inconclusive Reaction =	Development of color combinations other than those specified for a positive reaction, including one test positive and the other test negative

5.5.1.6.9 Reporting Results

5.5.1.6.9.1 Report positive test results as "blood was indicated…"

5.5.1.6.9.2 Report negative test results as "no blood was detected..."

5.5.1.6.9.3 Report inconclusive test results as "tests for blood were inconclusive…"

5.5.2 LUMINOL TEST (Reference 5, Appendix B)

5.5.2.1 Safety Considerations

5.5.2.1.1 Sodium perborate - Caution! Harmful if swallowed, inhaled or absorbed through skin!

5.5.2.1.2 Aminophthalhydrazide (luminol) - Caution! Irritant! Emits toxic fumes under fire conditions!

5.5.2.2 Materials and Equipment

5.5.2.2.1 Spray bottle (must contain no metal parts as the luminol reacts with some metals)

5.5.2.2.2 50 ml graduated cylinder

5.5.2.2.3 Balance

5.5.2.2.4 Weigh boats or weigh paper

5.5.2.2.5 Spatula

5.5.2.2.6 Ziploc bags (optional)

5.5.2.3 Stock Solutions

5.5.2.3.1 Solution A

- 0.7 g Sodium perborate
- 50.0 ml Distilled water
- Mix above ingredients until thoroughly dissolved. USE IMMEDIATELY! DO NOT STORE.

5.5.2.3.2 Solution B

- 0.1 g Aminophthalhydrazide (luminol)
- 5.0 g Sodium carbonate
- 50.0 ml Distilled water
- Mix the above ingredients until thoroughly dissolved. USE IMMEDIATELY! DO NOT STORE.

NOTE: The dry chemicals in Solutions A and B can be weighed out and placed in appropriately labeled zip lock bags and stored in the dark at room temperature. Each bag must be labeled with the date prepared, the expiration date, the initials of the person who prepared each package, and the amount of distilled water to be added. Water can be added when needed.

5.5.2.4 Minimum Standards and Controls

5.5.2.4.1 Test a positive reagent control (known bloodstain) and a negative reagent control (distilled water) to ensure that the reagents are working properly. The results of this testing must be documented in the case file.

5.5.2.4.2 If either control does not give the expected result, do not proceed with testing evidence samples until the problem has been resolved as demonstrated by testing another set of positive and negative reagent controls and achieving the expected results with both controls.

5.5.2.5 Luminol Procedure

5.5.2.5.1 WHEN THE TEST IS READY TO BE CONDUCTED, mix equal parts of solutions A and B and place in a sprayer bottle.

5.5.2.5.2 Under darkened conditions, immediately after mixing equal parts of solutions A and B, spray the positive and negative controls to ensure that the reagents are working properly. If both controls give the expected results, proceed with spraying the area of interest. Document results in the case file.

5.5.2.5.3 Areas containing blood will luminesce immediately and maintain a sustained glow.

5.5.2.5.4 Mark luminescent areas for subsequent testing with the Combined Phenolphthalein-Tetramethylbenzidine Test.

5.5.2.5.5 Interpretation

5.5.2.5.5.1 Positive Reaction = Immediate luminescence

5.5.2.5.5.2 Negative Reaction = No luminescence

5.5.2.5.5.3 Inconclusive Reaction = Slow and/or weak luminescence

DETECTION OF BLOOD STUDY QUESTIONS

1. What is blood and what is it composed of?
2. What is the purpose of blood in the body?
3. What is the PTMB test?
4. When is the PTMB test performed?
5. What is the mechanism behind the PTMB test?
6. What is the purpose of each chemical used for the PTMB testing?
7. If an oxidizer, such as potassium permanganate, was tested with the chemicals used for PTMB testing, what reaction would be expected and why?
8. What is the benefit of using the combined PTMB chemical test?
9. Which PTMB reagent works best in the acidic environment and which works best in the basic environment?
10. What does a positive PTMB result tell you?
11. What would you do if your P test was positive but the TMB test was negative?
12. What action would you take if your negative control was positive?
13. What is the mechanism for luminol?
14. When is luminol used?
15. What does a positive luminol reaction look like and what does it tell you?
16. You get a call from an investigator requesting luminol. How do you handle it? What are the essential questions you need to ask? How do you instruct him to use it?
17. You get a call from an investigator asking if luminol can be used to examine the back yard where it is believed that a husband shot his wife two weeks ago. What do you tell him?
18. You get a call from a patrol officer saying that he is processing the scene of a B&E where he sees blood on the broken window. He's never done this before. How do you advise him to proceed?
19. You get a "supercan" trashcan for a case in which the victim's body was found in the supercan itself. What do you do with it?
20. What is the purpose a positive control?
21. Name 2 presumptive tests for blood and 2 confirmatory tests for blood not used by the Department.

CHECKLIST FOR THE DETECTION OF BLOOD

Name of Trainee:_____

1. Preparation of the Combined Phenolphthalein-Tetramethylbenzidine and Luminol test reagents.

   Date:_____ Training Coordinator:_____

   Comments:_____

2. Completion of the Combined Phenolphthalein-Tetramethylbenzidine and Luminol tests for:

   Bloodstain dilutions (1:10, 1:100, 1:250, 1:500, 1:750, 1:1000, 1:2000).

   Date:_____ Training Coordinator:_____

   Comments:_____

   Aged bloodstains (5 minimum).

   Date:_____ Training Coordinator:_____

   Comments:_____

   Bloodstains subjected to various contaminants (20 minimum).

   Date:_____ Training Coordinator:_____

   Comments:_____

   Substances reported to give false positive reactions.

   Date:_____ Training Coordinator:_____

   Comments:_____

   Accurately tested at least 10 unknown stains (provided by training coordinator or designee).

   Date:_____ Training Coordinator:_____

   Comments:_____

3. Trainee has developed a thorough understanding of the theory behind and practical application of the various color tests for blood, including the sensitivity and specificity of the reagents, specificity and limitations of the various methods used, and the purpose and use of controls.

   Date:_____ Training Coordinator:_____

   Comments:_____

4. Trainee has developed a basic understanding of the use of presumptive and confirmatory tests and is familiar with tests in each category.

   Date:_____ Training Coordinator:_____

   Comments:_____

5. Notebook is organized and complete.

   Date:_____ Training Coordinator:_____

   Comments:_____

6. Trainee has participated in a mock trial and/or practical or oral examinations. Performance was satisfactory.

   Date:_____ Training Coordinator:_____

   Comments:_____

7. Trainee has read and understands all applicable literature.

   Date:_____ Training Coordinator:_____

   Comments:_____

♦END